Incorporation of Cro thermal water in a dermocosmetic formulation: cytotoxicity effects, characterization and stability studies and efficacy evaluation

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Abstract

OBJECTIVE: Development of cosmetic formulations to provide a controlled release of hydrophilic active compounds from mineral medicinal waters constitutes an attractive challenge. The objective of this study was the development and the characterization of a dermocosmetic gel formulation with Cró thermal water, from Beira Interior of Portugal, as a major functional ingredient.

METHODS: Concentrations of mineral chemical elements of Cró thermal water were previously determined by inductively coupled plasma-optical emission spectrometry and cytotoxicity assays using thermal water were carried out on normal human dermal fibroblasts (NHDF) cells. Then, the Cró thermal water was included (95%) in a developed gel formulation that was characterized through rheological and texture analysis and submitted to stability assays during 30 days. The effects on the skin volunteers, namely skin pH, the degree of hydration, transepidermal water loss and skin relief, were evaluated through non-invasive biometric techniques. A gel formulation including purified water as used as control.

RESULTS: Cró thermal water is rich on several chemical elements in particular sodium, silica, potassium and calcium besides some trace elements, with important functions for the skin. NHDF cells adhered and proliferated in the presence of thermal water confirming the biocompatibility of the major component of the gel formulation. The developed gel formulation based on thermal water resulted in an improvement of textural parameters, comparing with the purified water-based one. Significant improvements in the cutaneous biometric parameters (degree of hydration, transepidermal water loss and skin relief) of volunteers were also registered for the gel formulation containing thermal water.

CONCLUSION: This study demonstrated for the first time the potential benefits of Cró thermal water in a gel formulation to be used in cosmetic and dermatological applications.

Keywords: Cell culture; Dermocosmetic; Formulation/stability; Portuguese Cró Thermal water; Skin barrier.

Introduction

Cosmetics are products intended to improve the appearance and/or protecting the skin with the help of excipients and active ingredients adapted to different skin types and to keep skin in good condition according to Regulation European Commission (EC) 1223/2009. When skin is sensitive or presents disorders, are frequently associated sensations of itching, redness or tightness [1]. In these cases, conventional cosmetics are not adequate, and it is necessary to use formulations adapted to specific needs, free of substances that may cause irritations and other adverse reactions, or including active compounds with beneficial effects on skin condition. To get the balance and the hydration of the skin, and to achieve the cutaneous welfare, it could be used mineral thermal waters that are currently included on formulations for medicinal purposes [2, 3]. In addition, since mineral medicinal waters are rich in minerals and oligoelements with proved dermatological indications, it can be considered as a useful raw material for dermocosmetic formulations, which are widely recognized for the capacity to encapsulate, stabilize, carry and deliver these elements [4-6]. Indeed, the development of dermocosmetic formulations to provide a prolonged release and better skin penetration of hydrophilic active compounds from thermal waters is an attractive challenge [7].

Mineral medicinal waters are commonly used for the treatment of chronic dermatoses, mainly atopic dermatitis and psoriasis which are often associated with skin dryness and pruritus, increasing the quality of life and compliance in patients [8-11]. The most used in dermatological thermal treatments are the sulfurous and the chlorided bromo-iodic waters [12], the sulfated hypotonic ones [13] or even bicarbonate magnesium rich in fluoride [14]. Besides these types of waters, it is possible to use those that possess special mineral elements such as silica, calcium, magnesium, zinc, selenium and other trace elements such as boron and manganese [4]. Furthermore, the different properties of

mineral medicinal waters, such as detergent, anti-inflammatory, keratoplastic, antipruriginous, and antioxidant can be used together with cosmetics and are associated with the interaction between its components and the skin structure [8, 15, 16]. On the other hand, one of the most important defence functions of the skin is to maintain the homeostasis through the prevention of uncontrolled water loss and the permeability of ions and serum proteins between organism and surrounded environment [17]. The ability of the skin to hold water is primarily related to the *stratum corneum* (SC), which plays the role of a barrier to water loss [18]. The control of skin hydration level has a high impact on mechanical and optical properties of skin and contributes to preserving the barrier function as well as the regulation and enzyme activation on the flaking process. The deviation of this process affects barrier function and results on dry skin [19]. In this sense, hydration evaluation is one of the most relevant bioindicators of skin health and one of the most relevant parameters to be monitored in the development of new products in cosmetic research [16, 20-22].

The Cró thermal water is a medium mineral thermal water, from a *SPA* of Beira Interior region (Portugal), with a historical and registered indication for dermatological disorders by National Portuguese Health Authority [12, 15, 16]. Thus, the aim of the present work was the development of a dermocosmetic formulation, namely a hydrophilic gel, containing Cró thermal water as a major functional ingredient, and compared with a control gel formulation prepared with purified water.

Material and Methods

Test materials

All chemicals used were of analytical reagent grade. Carbopol 940® (carbomer), propylene glycol and lavender essential oil were purchased from Acofarma (Terrassa,

Spain). Triethanolamine (85%) was obtained from Farma-Quimica Sur S.L. (Malaga, Spain), imidazolidinyl urea was obtained from Guinama (Valencia, Spain) and green mint dye from Sancolor (Barcelona, Spain). Dulbecco's modified Eagle's medium-F12 (DMEM-F12) was purchased from Sigma-Aldrich (Darmstadt, Germany). [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) was purchased from VWR International (Ohio, USA).

Cró thermal water was obtained from its natural source and purified water was prepared by a Puranity TU 6 system from VWR (Leuven, Belgium) with a specific conductivity of less than 0.1 μS cm^{-1}.

Determination of the chemical composition of the Cró thermal water

Thermal water was acidified with purified nitric acid (0.1%, v/v). The concentrations of major and minor elements, namely boron (B), calcium (Ca), potassium (K), magnesium (Mg), sodium (Na) and silica (Si), were determined by inductively coupled plasma-optical emission spectrometry (ICP-OES) with an Optima 7000 DV spectrometer (Perkin Elmer, Norwalk, Connecticut, USA) equipped with an Échelle monochromator and a dual CCD detector, with backlighting and a cooling system; Mira Mist nebulizer and cyclonic spray chamber were used. The analyte signals were recorded at the following wavelengths: 249.677 nm (B); 317.933 (Ca); 766.49 nm (K); 285.213 nm (Mg); 589.592 nm (Na); and 251.611 nm (Si). The concentrations of elements present at trace and ultra-trace levels, namely manganese (Mn) and zinc (Zn) were determined by inductively coupled plasma mass spectrometry (ICP-MS) with a magnetic sector Thermo Finnigan Element 2 spectrometer. A glass concentric nebulizer, a Scott spray chamber and a secondary electron multiplier detector were used. On each sample, a minimum of triplicate 180 s analyses was conducted following a 50 s uptake and stabilization period.

Between samples, the nebulizer system was rinsed for 2 min with 2% nitric acid, which eliminated carry-over and reconditioned the sampler cone. The following isotopes of the investigated elements were monitored: ^{55}Mn in medium resolution and ^{67}Zn in low resolution.

During both ICP-OES and ICP-MS analyses, sets of instrumental blank and calibration verification checks were run at frequent intervals. The accuracy of the results was verified by analysing a standard reference material (NIST 1640, "Trace elements in natural water").

Proliferation of human fibroblast cells in the presence of Cró thermal water

In order to evaluate the growth of cells in the presence of Cró thermal water, Normal Human Dermal Fibroblasts (NHDF) bought from PromoCell (Labclinics, S.A.; Barcelona, Spain) were used as a cell model once they are widely used for the evaluation of the cytotoxicity of cosmetic formulations [23-25]. To assess cell proliferation in the presence of thermal water, NHDF were seeded in 96-well plates 2×10^4 cells/well with DMEM-F12 prepared with thermal water and supplemented with heat-inactivated FBS (10% v/v) and 1% antibiotic/antimycotic solution. After that, the cells were kept in culture at 37 °C in a 5% CO_2 humidified atmosphere. In control groups, the DMEM-F12 was prepared with MilliQ® water Ethanol 96% was added to cells to be used as positive controls (dead cells), and medium prepared with purified water was used as negative controls (live cells). Cell growth was monitored by using an inverted light microscope (Optika, Bergamo, Italy) equipped with an Optikam B5 digital camera (Optika, Bergamo, Italy) after 1, 3 and 9 days.

Characterization of the cytotoxic profile of Cró thermal water

Additionally, cell viability was assessed through the reduction of the MTS into a water-soluble brown formazan product. Briefly, from NHDF cells seeded at a density of 2×10^4 cells/well (96 wells plates) and grown in DMEM-F12 prepared with thermal water ($n = 5$), cell viability was assessed after 1, 3 and 9 days. The positive and negative control groups were performed as described in the previous section. Then, the culture medium from each well was removed and replaced with a mixture of 100 μL of fresh culture medium and 20 μL of MTS reagent solution. The cells were further incubated for 4 h at 37 °C under a humidified atmosphere with 5% CO_2. The absorbance of the formazan was measured at 490 nm using a microplate reader (Multiskan GO, Thermo Scientific, Ratastie, Finland). The extent of cell viability was expressed as the percentage of viable cells in comparison with negative control cells.

Preparation of gel formulation

The gel was prepared by addition, under stirring, of the water (thermal or purified) to Carbopol 940® (0.5 %) and imidazolidinyl urea (0.2 %), with propylene glycol (5.0 %) and neutralized with triethanolamine (q.s. to pH = 5.5), after the addition of the green mint dye (q.s.) and the flavouring agent (lavender essential oil, 2.5 %). After gel preparation, it was transferred to a glass container, protected from light, before any further studies.

Characterization and stability of the gel formulation

The characterization and stability studies included visual evaluation of the organoleptic characteristics (colour and odour), pH measurement, rheological studies and texture analysis. The pH was measured using a potentiometer (Mettler Toledo, Schwerzenbach,

Switzerland). The rheological studies were conducted with a rotational viscometer (Fungilab Alpha series L, Barcelona, Spain). Spindle number L4 was employed for the following speeds: 1.5, 3.0, 4.0, 5.0, 10.0, 12.0, 20.0 and 30.0 rpm at 20 ± 2 °C. For each speed, the apparent viscosity values (mPa.s) were recorded. The textural analyses were performed at 20 ± 2 °C using a texturometer (Stable Micro Systems TA.XTPlus, Surrey, United Kingdom); the following parameters were investigated: firmness, adhesiveness and spreadability. The firmness and the adhesiveness were evaluated by carrying out a penetration test using a load cell of 5 kg, a compression disc (40 mm diameter), a penetration depth of 15 mm, a test speed of 2 mm/s and a trigger force of 30 g. After penetrating the sample, the probe returned to the initial position. From the obtained graph force *versus* distance, the maximum force (g) (firmness) and the negative area (g × s) (adhesiveness) were calculated. The spreadability evaluation was performed using a TTC spreadability test probe, a penetration depth of 15 mm and a test speed of 3.0 mm/s. In this test, the sample is placed into the female cone, avoiding the incorporation of air. The sample surface is leveled, the probe placed at a defined position (25 mm) and the assay start with the male cone's downward movement, which compresses the sample, promoting its scattering between the surfaces of the two cones.

All these measurements were performed after 1, 15 and 30 days of storage of gel in the dark at 20 ± 2 °C and relative humidity (RH) 50 ± 10 % and were performed in triplicate.

Efficacy evaluation of the developed gel formulation through cutaneous biometry

Twenty healthy human volunteers (five men and fifteen women) participated in the study to evaluate the effect of the formulation on different parameters, such as skin pH, degree of hydration, transepidermal water loss (TEWL) and skin relief. All the volunteers participating in the study provided their informed written consent. The volunteers rested

at least 20 min, so that their blood circulation can regain a normal level after possible physical exercise. The skin area (forearm) were not covered with clothes during the acclimatization time. They were also instructed to not apply any cosmetic or wash the forearms before and during the test. The volunteers were randomly divided in two groups, that applied thermal or purified water-based gel formulation, with regards to similarities in their skin measurements. The skin surface pH values are between range of 5.0 and 6.0, considered the range that preserves the physiologic acid nature of the skin. The hydration levels are both between 30 and 40 arbitrary units (AU), that correspond to a dry skin and TEWL values are close to 9 g/h/m^2 and then lower than 10 g/h/m^2, that correspond to very healthy skin condition.

Skin pH (Skin- pH-Meter® PH 905), hydration (Corneometer® CM 825) and TEWL (Tewameter® TM 300) were determined using a Multi Probe Adapter MPA 6® equipment (Courage-Khazaka, Köln, Germany). Those determinations were performed in quintuplicate. The skin surface images were obtained with a Visioscan® VC 98 equipment (Courage-Khazaka). The determinations were performed in the forearm before gel application and 30 and 60 min after the application of 1.50 g of the gel formulation.

Statistical Analysis

The statistical analysis of the viscosity, skin pH surface, hydration and TEWL results obtained was performed using 2-way ANOVA followed by Bonferroni post-tests. A p value lower than 0.05 ($p < 0.05$) was considered statistically significant.

Results and discussion

Chemical composition of Cró thermal water

Table I reports the concentrations of the chemical elements of Cró thermal water. The chemical composition of Cró thermal water revealed the presence of sodium, silica, calcium, potassium and some trace elements, that play important roles in skin homeostasis and regeneration, as summarized in Table I. Also in previous studies, this thermal water has been characterized by the presence of total sulphur (16.9 in I_2 0.01 N), with noteworthy functions regarding the skin, such as cell regenerator, antioxidant, antibacterial and antifungal activity, as well as bicarbonate (157 mg/L), chloride (33 mg/L) and sulphate (14.1 mg/L) anions [15].

The dermatologic therapeutic indications invoked by the presence of such elements were recently reviewed by M.Z. Karagülle *et al.* [27], and can be related to the beneficial effects of the incorporation of mineral thermal waters on specific formulations for topical applications, namely cosmetics.

Proliferation and cytotoxicity evaluation of the Cró thermal water

The Cró thermal water promotes the NHDF adhesion and proliferation, with no microscopic differences registered when compared to the negative control after 1, 3 and 9 days (Fig.1). Dead cells with their typical spherical shape were observed in the positive control (ethanol treated cells), as expected.

To further assess the effects of thermal water on cell viability, MTS assay was also performed. This assay showed that cells remained viable when were seeded with culture medium prepared with thermal water after 1, 3 and 9 days of incubation (Fig. 2). The results showed the biocompatibility of the Cró thermal water.

Characterization and stability studies of the developed gel formulation

The developed dermocosmetic formulation (hydrophilic gel), comprising more than 90% of thermal water was compared with a gel formulation with identical composition but using purified water (instead of thermal water) as a control. During the period of storage (until 30 days) at 20 ± 2 ºC and RH 50 ± 10 % the gel formulation, either thermal or purified water-based showed adequate organoleptic properties (translucid aspect and lavender flavour). The pH of the formulation was kept around 5.5 during the storage period. Relatively to rheological profiles, despite it were observed some statistical significant differences between thermal vs. purified water and along the 30 days of the storage, a similar profile in which was observed a decrease of viscosity with the increase of rotation speed, as it can be seen in Fig. 3 and in Table II. These formulation characteristics are important factors in the development and final behaviour of semisolid formulations [28, 29] and results revealed that the incorporation of thermal water does not greatly affect its viscosity.

The gel prepared with thermal water showed lower firmness (Fig. 4.A) and adhesiveness (Fig. 4.B) than the control gel prepared with purified water during 30 days of storage. Spreadability values (Fig. 4.C) were lower for the gel with thermal water than the gel with purified water. Therefore, the thermal water-based gel is expected to be easier to apply onto the skin and exhibits favourable adhesive properties, allowing a sustained release of active compounds. In general, texture analysis of gel formulation showed that exhibit acceptable characteristics for skin topical application [30,31].

Efficacy evaluation of the developed gel formulation through cutaneous biometry

The quantification of parameters such as skin pH, SC hydration and TEWL is essential for the integral evaluation of the epidermal barrier status. In the present work, cutaneous

biometry as a non-invasive *in-vivo* approach was used to monitor the skin barrier physical properties in accordance with literature [32].

Results showed that after gel application, the pH of the skin exhibited statistically significant differences between the groups that applied the purified water and thermal water-based formulations (Table III). However, for both formulations and considering the range of pH between 5.52-5.90, is considered the preservation of the physiologic acid nature of the skin [33]. This fact is especially relevant as the acidic milieu plays a central role for the epidermal permeability barrier homeostasis, restoration of the disrupted barrier, and non-specific antimicrobial defence of the skin [34].

The hydration degree was higher, both after 30 or 60 min of application of thermal water-based gel formulation, when compared with the purified water-based one and a statistically significant increase was found after 30 min of application of thermal water-based formulation in comparison with the initial hydration degree. This results confirm the improvement of the incorporation of Cró mineral medicinal water in the formulation, once is related to the the maintenance of water content of the SC that is determinant for permeability, its mechanical properties, as well as the regulation of hydrolytic enzymes involved in the process of normal corneocyte desquamation [21].

TEWL is related to permeability barrier status under normal, experimentally perturbed, or diseased conditions being used to assess the homeostasis of the permeability barrier but also indirectly to predict the influence of topically applied substances on the skin [35]. For TEWL was registered statistical significant decrease after 30 min with the application of the thermal water-based gel (Table V) which suggests that this formulation could have some occlusive effect on skin barrier function.

The skin relief was evaluated by the analysis of the images that skin structure, the dryness level and the real topography of skin surface. The Surface Evaluation of the Living Skin

(SELS) parameters were registered taking into account the images obtained by the Visioscan® software (Fig. 5 and Table VI), namely roughness (SEr), scaliness (SEsc) and kurtosis parameter (Rku). In general, Bbetter results were obtained for the gel formulation containing thermal water, as it was observed higher SEr (lesser roughness), lower SEsc (lower scaliness) (Table VI) and Rku values close to 3 (higher smoothness).

Conclusions

This preliminary work shows beneficial effects on skin when the Cró thermal water was used in a gel dermocosmetic formulation, which itself constitutes a noteworthy achievement when general skin care is needed. Furthermore, it opens promising perspectives in future utilization in cosmetics development as well as in the dermatological fields. In this sense, the vehiculation of this thermal water in dermoscosmetic formulations is envisioned as a potential tool for the treatment of dermatological diseases.

Conflicts of interest

The authors have declared no conflict of interest.

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